Video Article

Streamlined Single Cell TCR Isolation and Generation of Retroviral Vectors for *In Vitro* and *In Vivo* Expression of Human TCRs

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Abstract

Although, several methods for sequencing of paired T cell receptor (TCR) alpha and beta chains from single T cells have been developed, none so far have been conducive to downstream *in vivo* functional analysis of TCR heterodimers. We have developed an improved protocol based on a two-step multiplex-nested PCR, which results in a PCR product that spans entire variable regions of a human TCR alpha and beta chains. By identifying unique restriction sites and incorporating them into the PCR primers, we have made the PCR product compatible with direct sub-cloning into the template retroviral vector. The resulting retroviral construct encodes a chimeric human/mouse TCR with a mouse intracellular domain, which is functional in mouse cells or in *in vivo* mouse models. Overall, the protocol described here combines human single cell paired TCR alpha and beta chain identification with streamlined generation of retroviral vectors readily adaptable for *in vitro* and *in vivo* TCR expression. The video and the accompanying material are designed to give a highly detailed description of the single cell PCR, so that the critical steps can be followed and potential pitfalls avoided. Additionally, we provide a detailed description of the cloning steps necessary to generate the expression vector. Once mastered, the whole procedure from single cell sorting to TCR expression could be performed in a short two-week period.

Video Link

The video component of this article can be found at https://www.jove.com/video/55379/

Introduction

The T cell receptor (TCR) dictates T cell fate decision during development, steady state/homeostasis, and antigenic stimulation in periphery[1,2,3]. Recent expansion of deep sequencing technologies has uncovered a previously underappreciated TCR diversity within antigen specific T cell responses. TCR diversity suggests a potential for functionally broad T cell responses. In order to integrate the TCR sequence repertoire analysis with TCR functional assays, the sequencing approaches should be designed to be compatible with experimental systems and *in vivo* models utilized for subsequent functional analysis of select TCRs. We have developed an efficient approach for human TCR sequence isolation and streamlined sub-cloning into a chimeric human/mouse TCR template vector compatible with TCR expression in HLA-humanized mice[4]. Isolation of corresponding alpha and beta chains of heterodimeric TCRs requires PCR amplification of both chains from a single cell. Although, several single-cell TCR cloning protocols have been developed and utilized, none so far have been easily compatible with high-throughput streamlined direct cloning of unknown Vα/Vβ TCRs into retroviral vectors necessary for re-expression *in vivo*[4,5,6,7]. Previous studies have utilized two major approaches, either to selectively amplify a limited portion of the TCR sufficient to extrapolate the sequence, or to amplify the entire TCR sequence[4,5,6,7]. Downstream functional analysis of TCR sequences obtained via the first approach requires costly *in silico* assembly and *de novo* construction of the TCR. While the second approach provides the complete TCR sequence, the human constant region is not compatible with *in vivo* expression of the cloned TCRs in mouse models. Our approach is specifically designed to be compatible with *in vivo* functional analysis of TCRs in mouse models. We developed an efficient and streamlined single cell PCR protocol that allows for direct sub-cloning of PCR fragments into the template expression vector.

Our approach utilizes a highly sensitive multiplex-nested PCR reaction that is performed in two steps. In the first multiplex reaction step a pool of 40 primers specific for all the V-beta chains and a pool of 44 primers specific for all the V-alpha chains are used to amplify the TCR-alpha or TCR-beta without the prior knowledge of the sequence (**Table 1**). The forward primer has an adaptor sequence, which is incorporated into the 5' of the PCR product. The reverse primer is based on the constant region of the TCR. We have identified unique restriction sites that are absent from human variable or junction TCR regions, and incorporated these into newly redesigned TCRα and TCRβ primers (**Figure 1**). In the second nested reaction, a primer specific for the adaptor sequence and a nested reverse primer within the constant region are used to further amplify the

TCR chains with increased specificity (**Table 1, Figure 1**). After single cell isolation, two rounds of PCR (first reaction with a pool of both Vα and Vβ primers, and second with adaptor primers) result in a PCR product that can be directly sub-cloned into the template retroviral vector. The final TCR construct will encode, in a single open reading frame (ORF), human variable regions combined with mouse constant regions connected by the 'self-cleaving' protein sequence, P2A (hVα-mCα-P2A-hVβ-mCβ)[8]. The P2A sequence has been used in multiple systems, and has been extensively tested specifically for the expression of TCRs[8,9,10,11]. Although, after translation most of the P2A sequence remains attached to the C-terminus of the alpha chain connected by a flexible linker, while the beta chain signal sequence has an additional proline, this modification has no detrimental effect on TCR function. The mouse constant region is used in the construct instead of human to avoid potential altered interactions with downstream signaling components when re-expressed in mouse cells. The single ORF will result in stoichiometric separate expression of alpha and beta chains[9,11]. The current protocol is based on reagents and approaches that are widely available, and is designed to be performed in a streamlined and efficient fashion. Although we have specifically used this technique to assay TCRs from self-reactive T cells implicated in autoimmune diabetes, we anticipate that this protocol can be widely applicable for identification and functional assessment of human TCRs specific for autoimmune epitopes, cancer epitopes, or responses to pathogens and vaccines.

Protocol

1. Identify the T cell population of interest.

NOTE: Antigen specific proliferation in combination with cell division dye (like Carboxyfluorescein succinimidyl ester, CFSE) can be used to isolate T cells based on their proliferation in response to antigenic stimulation. If starting from PBMCs, a 7-day *in vitro* expansion should be sufficient to identify an antigen specific CFSE low population[12].

1. Mix PBMCs 1:1 with MHC-matched feeder cells, and label with 5 µM CFSE cell division dye.
2. Plate 2-2.5 x 10^5 cells per well in a 96-well round-bottom culture plate in 200 µL 10% FCS complete RPMI media.
   NOTE: Complete RPMI contains 2 mM l-glutamine, 1 mM sodium pyruvate, 100 µM MEM nonessential amino acids, 5 mM HEPES, 5.5 × 10^{-5} units of 2-mercaptoethanol, 100 U mL^{-1} penicillin and 100 µg mL^{-1} streptomycin.
3. Stimulate cells with 25 µM peptide antigen.
4. On the fourth day of culture, carefully remove and replace 100 µL of media.
   NOTE: A more optimal approach for antigen-specific T cell identification is the use of peptide-HLA tetramers[13]. Tetramer staining allows for simultaneous assessment of antigen specificity, as well as HLA-restriction.

2. Set up the single cell sort.

1. Perform all procedures in a hood or a designated area free of amplified template. Aliquot the reagents to avoid contamination, clean all work surfaces and, if feasible, equipment with 10% bleach prior to PCR setup. Use filter tips, RNAse and DNAse free reagents and plastics in all the PCR and vector cloning steps. Key reagents can be found in the **Table of Materials**.
   NOTE: The multiplex PCR reaction is highly sensitive, and low levels of contamination can result in amplified product.
2. Generate the reverse transcription master mix by combining 10x RT reaction buffer, 25X DNTPs, 9 U RT enzyme, 0.01% Triton-X, 0.7 U RNAse inhibitor, and 383 nM of each RT-TCR primer (listed in **Table 1**) in a sterile pipetting reservoir. For each 96-well plate, make up enough master mix for 10 additional reactions (total 106), to compensate for loss of liquid during pipetting. See **Table 2** for reaction components and amounts per well.
3. Prepare a 96-well PCR collection plate for cell sorting by pipetting 6 µL of the reverse transcription master mix per well with a multichannel pipette. Keep the plate on ice or in a cold block.
4. Label cells with antibodies to cell surface markers (such as CD4 and CD3, or CD8 and CD3, each at 2 µg/mL, in combination with cell division dye or tetramer staining).
5. Sort T cells at one cell per well, skipping the twelfth row, which will serve as a negative control.
   NOTE: The efficiency of the sort will depend on precise collection plate alignment within the plate holder, as well as maintaining the cells and plates at a low temperature prior to cDNA reaction. Collection plates should be kept on ice at all times, except during sorting, when they are fixed within the cooling plate. Not all sorters are equipped with a temperature regulated plate holder; however, the use of one is strongly recommended. Since the sort generally takes about 10 - 15 min per plate, there is an increased potential for RNA degradation if the plate is not maintained at a low temperature.
6. As a positive control, add a larger number of cells (100 cells) manually with a pipette to one of the wells at this stage. Alternatively, previously isolated RNA from pooled cells can be used as a positive control at 10 ng per well.
   NOTE: Pipette purified control RNA with precision and care in order to avoid cross-contamination of other wells and false positive results.
7. Seal the plate with adhesive film, and spin down at 1000 x g for 5 min at 4 °C.
8. Immediately transcribe RNA to cDNA by incubating the plate at 25 °C for 10 min, 37 °C for 45 min, and 85 °C for 10 min.
   NOTE: To ensure efficient PCR reaction, it is recommended that the first PCR reaction be performed the same day. Alternatively, the transcribed plates can be kept in -80°C for up to 2 months.

3. Perform multiplex-nested PCR

NOTE: Prepare all master mixes in a clean template-free area to avoid contamination.

1. Reconstitute each variable region primer to 100 µM with DNAse and RNAse free H_2O. Next, combine 20 µL of each of the 44 primers to generate the V-alpha primer pool. To generate the V-beta primer pool combine 20 µL of each of the 40 V-beta primers plus 80 µL of DNAse and RNAse free H_2O. Primer sequences are listed in **Table 1**. Once pooled, the concentration of each primer in the mix is 2.3 µM.
2. Reconstitute the constant region primers to 100 µM stock solution with DNAse and RNAse free H_2O. Dilute the constant region primers to 10 µM working solution and aliquot for use.

3. Prepare two separate master mixes for TCR-alpha and TCR-beta amplification. Generate the master mixes by combining 5X buffer, 80 µM dNTPs, 3% DMSO, 2 µM primer pool (final concentration of 46 nM for each primer), 200 nM TCR-constant region reverse primer, 1 U DNA polymerase, and DNAse and RNAse free H_2O (**Table 2**). For each 96-well plate, make up enough master mix for 10 additional reactions (total 106), in order to compensate for loss of liquid during pipetting.
   NOTE: The calculations are based on the final PCR volume of 25 µL per well, where 22.5 µL master PCR mix is combined with 2.5 µL cDNA template.
4. Keep the plate with cDNA on ice or in a cold block. Use a multichannel to pipette 22.5 µL of TCR-beta reaction into a new 96-well PCR plate. Then use a multichannel to transfer 2.5 µL of cDNA into the PCR plate.
5. Use a multichannel to pipette 22.5 µL of TCR-alpha reaction directly into the plate containing the remainder of the cDNA.
6. Seal the plates with adhesive, gently vortex, and spin down at 1000 x g for 5 min at 4 °C.
7. Run the multiplex PCR reaction with the following cycles: 5 min at 95 °C followed by 34 cycles of 20 s at 95 °C, 30 s at 54 °C, and 1 min at 72 °C, followed by 7 min at 72 °C.
8. Carry out the nested PCR reaction in a final volume of 25 µL, using 2.5 µL of the multiplex PCR mixture as template.
   1. Reconstitute the internal and adaptor primers to 100 µM with DNAse and RNAse free H_2O. Make 10 µM aliquots for the working stock.
   2. Prepare two separate master mixes for TCR-alpha and TCR-beta amplification. Mix 5X buffer, 25X DNTP, 3% DMSO, 200 nM forward adaptor primer, 200 nM reverse nested primer, 1 U DNA polymerase, and DNA polymerase and nuclease-free H_2O for a final volume of 22.5 µL (**Table 2**). For each 96-well plate, make up enough master mix for 10 additional reactions (total 106), to compensate for loss of liquid during pipetting.
      NOTE: In the second PCR reaction use the 5X PCR buffer containing loading dye to facilitate agarose gel loading.
   3. Using a multichannel, pipette the master mixes for the TCR-alpha and TCR-beta reactions at 22.5 µL per well into two new 96-well PCR plates.
   4. Add the multiplex PCR reaction template at 2.5 µL per well.
      NOTE: The remaining first PCR reaction should be sealed, and stored at -20 °C to serve as a source of additional template, if it becomes necessary (see **step 4.3 Note**).
   5. Seal the plates, gently vortex, and spin down at 1000 x g for 5 min at 4 °C.
   6. Run the nested PCR reaction with the following cycles: 5 min at 95 °C followed by 34 cycles of 20 s at 95 °C, 30 s at 56 °C, and 1 min at 72 °C, followed by 7 min at 72 °C.
   7. Run 5 µL of the final reaction out on a 1% agarose gel containing ethidium bromide (including the negative and positive control wells).
      NOTE: The expected band should run at around 500 bp size. An example reaction is shown in **Figure 2**.
   8. Purify the remainder of the second reaction (20 µL) using a 96-well format PCR purification kit, elute with 15 µL of 70 °C H_2O per well, and perform Sanger sequencing. Use the appropriate TCR-alpha or TCR-beta adaptor forward primer for sequencing (**Table 1**).
      NOTE: Sequences can be analyzed with online available immunogenetics software.

4. Sub-cloning alpha and beta PCR chains.

NOTE: The pool of the forward primers utilized in the first reaction, and the reverse primers in the second PCR reaction are used to incorporate restriction sites. Therefore, the obtained alpha and beta chain PCR products are ready to be sequentially sub-cloned into the template retroviral vector (**Figure 1**).

1. Digest the purified PCR products from the beta nested reaction with BstbI and MfeI (**Table 3**).
   NOTE: Do not exceed 5 µg insert.
2. In parallel, digest the template vector with BstbI and MfeI (**Table 3**). Use 1-2 µg of the template vector per TCR. Run the digested vector on an agarose gel, and isolate the larger band (~7500 bp). Purify the vector with a gel DNA purification kit.
   NOTE: During the restriction enzyme digest, the template murine TCR-beta variable region is removed, allowing for the addition of the human TCR-beta variable region.
3. Purify the digested PCR products using a PCR purification kit.
   NOTE: Verify the DNA purification capacity of the columns included in the kit; use several columns if necessary. The minimum amount of a purified insert necessary for a successful ligation reaction is 50 ng at a minimum concentration of 10 ng/µL. If the purified insert amount is not sufficient for a successful ligation, the second PCR reaction can be repeated.
4. Treat the template vector with 10 U CIP enzyme for 1 h at 37 °C to prevent self-ligation, followed by heat inactivation for 10 min at 75 °C. Purify the DNA with a PCR purification kit (**Table 3**).
5. Ligate TCR-beta insert and vector using DNA ligase in a 20 µL total volume at 6 molar excess of the insert for 30 min at room temperature.
   NOTE: In total, the ligation reaction should contain about 150 ng of DNA (**Table 3**).
6. For transformations, mix 8 µL of the ligation reaction with 80 µL of chemically competent *E.coli* DH5α cells (**Table of Materials**) and incubate on ice for 30 min. Heat shock at 42 °C for 45 s. Add 500 µL Super Optimal broth with Catabolite suppression (S.O.C.) medium and shake for 1.5 h at 37 °C.
7. Plate out 250 µL of bacterial culture on an ampicillin containing Lysogeny Broth (LB) agar plate. Incubate the plate overnight (~16 h) at 37 °C.
8. The next day pick bacterial colonies, and grow them up in 3 mL LB medium containing ampicillin overnight. Isolate the plasmid DNA using DNA plasmid purification kit.
9. Confirm the successful insertion of the beta chain by a small-scale test-digest with the restriction enzymes BstbI and MfeI. In a 12 µL total reaction volume, combine 200-500 µg plasmid DNA, 0.25 µL of each enzyme, and 10X enzyme buffer (**Table 3**). Run the reaction out on a 1% agarose gel containing ethidium bromide to confirm the size of the insert band (~500 bp).
10. After confirming the presence of the insert, sequence the TCR-beta section of the vector using the IRES reverse primer (**Table 1**).
11. Following sequence confirmation of the TCR-beta insert, perform a similar process for insertion of the alpha variable region. Digest the purified PCR products from the alpha nested reaction, and the newly generated TCR-beta insert containing vector, with SnaBI and SacII.
    NOTE: During the restriction enzyme digest, the template murine TCR-alpha variable region is removed, allowing for the addition of the human TCR-alpha variable region.

12. Ligate alpha inserts into TCR vectors encoding the corresponding beta variable region using DNA ligase in a 20 µL total volume at 6 molar excess of the insert for 30 min at room temperature.
    NOTE: In total, the ligation reaction should contain about 150 ng of DNA (**Table 3**).
13. For transformations, mix 8 µL of the ligation reaction with 80 µL of chemically competent *E.coli* DH5α cells (**Table of Materials**) and incubate on ice for 30 min. Heat shock at 42 °C for 45 s. Add 500 µL S.O.C. medium and shake for 1.5 h at 37 °C.
14. Plate out 250 µL of bacterial culture on an ampicillin containing Lysogeny Broth (LB) agar plate. Incubate the plate overnight (~16 h) at 37 °C.
15. The next day pick bacterial colonies, and grow them up in 3 mL LB medium containing ampicillin overnight. Isolate the plasmid DNA using DNA plasmid purification kit.
16. Confirm the successful insertion of the alpha chain by a small-scale test-digest with the restriction enzymes SnaBI and SacII. In a 12 µL total reaction volume combine 200-500 µg plasmid DNA, 0.25 µL of each enzyme, and 10x enzyme buffer (**Table 3**). Run the reaction out on a 1% agarose gel containing ethidium bromide to confirm the size of the insert band (~500 bp).
17. After confirming the presence of the insert, sequence the TCR-alpha section of the vector using the MSCV2-forward primer (**Table 1**).
18. Once the alpha chain sequence is validated, verify the complete TCR insert by sequencing with TCR-beta-reverse and IRES-reverse primer (**Table 1**).

5. Verify TCR cell surface expression and specificity.

Note: HEK293T cells are used to test successful TCR chain paring and cell surface expression (**Figure 3A**)[8]. Peptide-MHC tetramer staining can also be performed on the transfected HEK293T cells to test for retention of antigenic specificity post TCR gene isolation (**Figure 3B**).

1. Plate out HEK293T cells in 12-well tissue culture plates at 1 x 10^5 cells per well in 1 mL complete DMEM media containing 5% FCS, and culture overnight at 37 °C. Plate out enough cells to have designated separate wells for each new TCR, control template TCR vector, CD3 vector only, control TCR vector only, and a non-transfected well.
   NOTE: Transfection with a fully mouse template TCR vector (mTCR-pMIA) in combination with mCD3εγδζ-pMIG vector, mTCR-pMIA vector alone, and mCD3εγδζ-pMIG vector alone are used as positive and negative controls.
   NOTE: Complete DMEM contains 2 mM L-glutamine, 1 mM Sodium Pyruvate, 100 µM Non-essential Amino Acids, 5 mM HEPES buffer, 5.5 x 10^{-5} units of 2-mercaptoethanol, 100 U/ml Penicillin/Streptomycin.
2. The following day, transfect the cells with 1 µg chimeric h/mTCR-pMIA vector, 1 µg mouse CD3εγδζ-pMSCVII-GFP (pMIG) vector, and a liposome-based transfection reagent following manufacturer instructions.
3. For each TCR, add 6 µL transfection reagent to 100 µL room temperature, serum-free DMEM. Briefly vortex, and incubate at room temperature for 15 min.
4. In separate 1.5 mL tubes combine 1 µg of TCR-pMIA vector with 1 µg mouse CD3εγδζ-pMSCVII-GFP (pMIG) vector, or 1 µg of each vector separate for controls.
5. Dropwise, add the transfection reagent/DMEM solution to the tubes containing the vector DNA. Incubate at room temperature for 15 min.
6. Dropwise, add the transfection reagent/DMEM/DNA solution to the HEK293T cells. Gently tap the plate to mix. Incubate at 37 °C for 24 h.
7. After 24 h replace the media, and keep cells in culture for additional 24 h.
8. Remove cells from the plate by vigorous pipetting, and stain the cells with anti-mouse CD3 antibody (2.5 µg/mL) to verify surface expression of the chimeric-TCR by flow cytometry.
9. In order to compare CD3 expression between cells that were transfected to a similar level, gate the analysis on cells expressing a similar high level of fluorescent reporter molecules (GFP for CD3 complex, and Ametrine for TCR expression vector). Optimally, the analysis should be gated on GFP+Ametrine+ cells within 10^4 to 10^5 fluorescent intensity (**Figure 3A**).
   NOTE: The level of CD3 expression in cells transfected with the chimeric construct should be comparable to the control mouse (original template) vector (**Figure 3A**). The generated TCR retroviral vectors are compatible with *in vivo* expression systems as previously described[4].

Representative Results

The efficiency of the multiplex-nested PCR reaction is checked in step 3.2.7 (**Figure 2**) by running out 5µL of the second reaction on an agarose gel. On average the efficiency of TCR-beta amplification is expected to be around 80%, while the efficiency of the TCR-alpha reaction is usually lower, at around 50%[4]. Only paired TCR-alpha and TCR-beta chains can be used for TCR expression; however, all PCR products could be sequenced to obtain TCR sequence and repertoire information.

Gene silencing will only allow a single TCR-beta chain to be expressed in a given T cell. However, a T cell is capable of rearranging a second TCR-alpha chain, and about 25% of T cells will expressing a second in-frame TCR-alpha chain [14]. It is expected that some proportion of the isolated TCR-alpha chains will be the secondary alpha instead of the "correct" alpha chain. The secondary alpha chain is often not an optimal binding partner with the TCR-beta chain; consequently, it is expected that not all cloned TCRs will form a stable complex and express on cell surface. Therefore, the proper TCR chain pairing and cell surface expression has to be confirmed by transfecting HEK293T cells (**Figure 3A**).

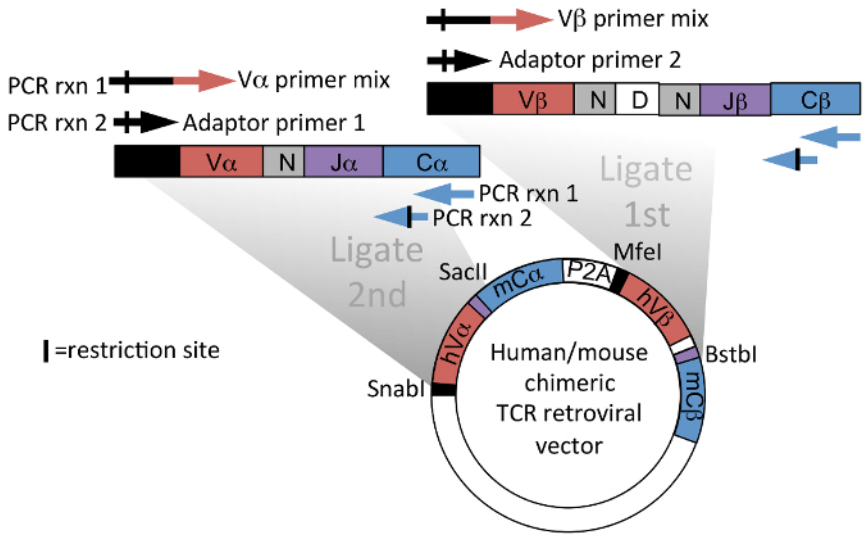

Figure 1. Streamlined multiplex-nested PCR and retroviral construct development from single T cells. Two rounds of PCR result in amplification of corresponding TCR alpha and beta chains. Restriction sites embedded into the primers allow streamlined sub-cloning and generation of human/mouse chimeric vector for expression in mouse cells. The template mouse vector can be used to easily switch out variable mouse regions for human, generating a chimeric human/mouse TCR retroviral vector compatible with expression in mouse cells. Please click here to view a larger version of this figure.

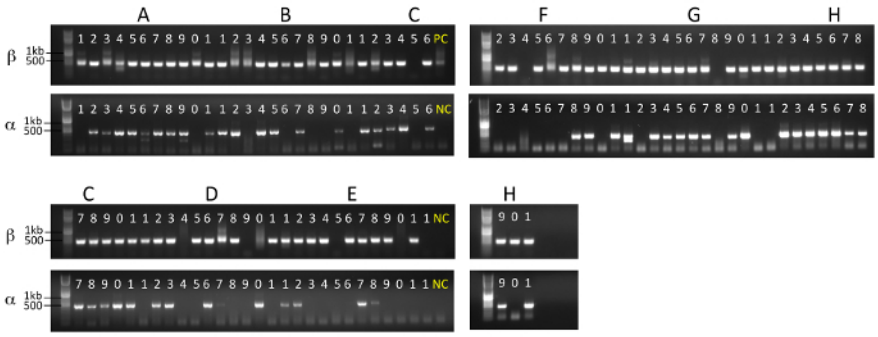

Figure 2. Example of single cell PCR. Multiplex nested PCR amplification of T cell receptor beta and alpha chains from single human T cells sorted into one 96-well plate. Shown are corresponding (top and bottom) PCR products from single cell amplified TCR beta and TCR alpha chains from human PBMCs. NC - no template control, PC - positive control. By running a small portion of the reaction on an agarose gel (5 µL), the efficiency of the single cell alpha and beta chain PCR reactions is confirmed prior to PCR product sequencing. Please click here to view a larger version of this figure.

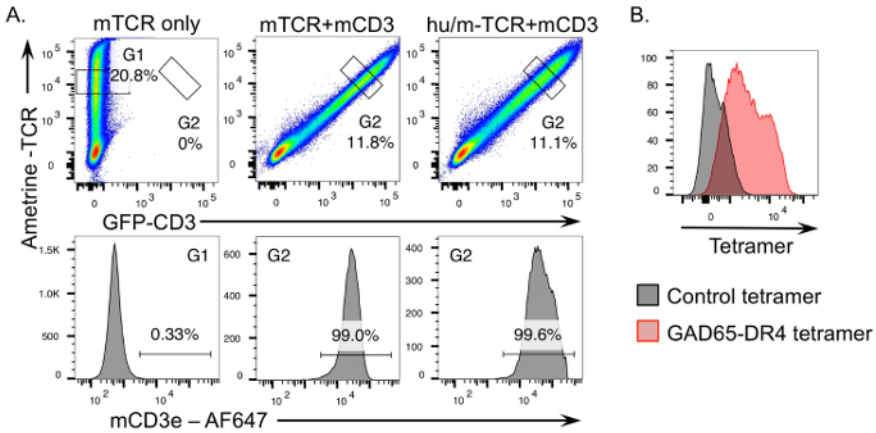

Figure 3. Verification of cell surface chimeric TCR expression and specificity. (**A**) HEK293T cell were transfected with mouse TCR or a human/mouse chimeric TCR in combination with mouse CD3εγδζ. Cells surface TCR expression was measured with anti-mCD3ε antibody. Gating strategy: First, the analysis is gated on Ametrine and GFP double positive cells (G2). In the case of single vector controls the gating has to be based on the single fluorescence (G1). Second, the cells from G1 and G2 are analyzed for the level of CD3ε expression. (**B**) TCR specificity is confirmed by tetramer staining of 293T cells with DRB1*0401:GAD115-127 tetramer. Analysis is gated on GFP+Ametrine+CD3ε + cells, as shown in (**A**). Please click here to view a larger version of this figure.

TARGET GENE	PRIMER ORIENTATION	SEQUENCE
Reverse Transcription		
TRAC cDNA		AGCTGGACCACAGCCG
TRBC1 cDNA		GAAATCCTTTCTCTTGACCATG
TRBC2 cDNA		GCCTCTGGAATCCTTTCTCT
TCR alpha PCR reaction 1		
TRAV1-1	Forward	CGGTTCAGCAGGAATGCCtacgtaATGTGGGGAGCTTTCCTTCTCTATGTTT
TRAV1-2	Forward	CGGTTCAGCAGGAATGCCtacgtaATGTGGGGAGTTTTCCTTCTTTATGTTTC
TRAV2	Forward	CGGTTCAGCAGGAATGCCtacgtaATGGCTTTGCAGAGCACTCTGG
TRAV3	Forward	CGGTTCAGCAGGAATGCCtacgtaATGGCCTCTGCACCCATCTCG
TRAV4	Forward	CGGTTCAGCAGGAATGCCtacgtaATGAGGCAAGTGGCGAGAGTGATC
TRAV5	Forward	CGGTTCAGCAGGAATGCCtacgtaATGAAGACATTTGCTGGATTTTCGTTC
TRAV6	Forward	CGGTTCAGCAGGAATGCCtacgtaATGGAGTCATTCCTGGGAGGTGTTT
TRAV7	Forward	CGGTTCAGCAGGAATGCCtacgtaATGGAGAAGATGCGGAGACCTGTC
TRAV8-1	Forward	CGGTTCAGCAGGAATGCCtacgtaATGCTCCTGTTGCTCATACCAGTGC
TRAV8-2	Forward	CGGTTCAGCAGGAATGCCtacgtaATGCTCCTGCTGCTCGTCCC
TRAV8-3	Forward	CGGTTCAGCAGGAATGCCtacgtaATGCTCCTGGAGCTTATCCCACTG
TRAV8-7	Forward	CGGTTCAGCAGGAATGCCtacgtaATGCTCTTAGTGGTCATTCTGCTGCTT
TRAV9-1	Forward	CGGTTCAGCAGGAATGCCtacgtaATGAATTCTTCTCCAGGACCAGCG
TRAV9-2	Forward	CGGTTCAGCAGGAATGCCtacgtaATGAACTATTCTCCAGGCTTAGTATCTCTGATACTC
TRAV10	Forward	CGGTTCAGCAGGAATGCCtacgtaATGAAAAAGCATCTGACGACCTTCTTG
TRAV12-1	Forward	CGGTTCAGCAGGAATGCCtacgtaATGATATCCTTGAGAGTTTTACTGGTGATCC
TRAV12-2	Forward	CGGTTCAGCAGGAATGCCtacgtaATGATGAAATCCTTGAGAGTTTTACTAGTGATCC
TRAV12-3	Forward	CGGTTCAGCAGGAATGCCtacgtaATGATGAAATCCTTGAGAGTTTTACTGGTG
TRAV13-1	Forward	CGGTTCAGCAGGAATGCCtacgtaATGACATCCATTCGAGCTGTATTTATATTCC
TRAV13-2	Forward	CGGTTCAGCAGGAATGCCtacgtaATGGCAGGCATTCGAGCTTTATTT
TRAV14	Forward	CGGTTCAGCAGGAATGCCtacgtaATGTCACTTTCTAGCCTGCTGAAGGTG
TRAV16	Forward	CGGTTCAGCAGGAATGCCtacgtaATGAAGCCCACCCTCATCTCAGTG
TRAV17	Forward	CGGTTCAGCAGGAATGCCtacgtaATGGAAACTCTCCTGGGAGTGTCTTTG
TRAV18	Forward	CGGTTCAGCAGGAATGCCtacgtaATGCTGTCTGCTTCCTGCTCAGG
TRAV19	Forward	CGGTTCAGCAGGAATGCCtacgtaATGCTGACTGCCAGCCTGTTGAG
TRAV20	Forward	CGGTTCAGCAGGAATGCCtacgtaATGGAGAAAATGTTGGAGTGTGCATTC
TRAV21	Forward	CGGTTCAGCAGGAATGCCtacgtaATGGAGACCCTCTTGGGCCTG
TRAV22	Forward	CGGTTCAGCAGGAATGCCtacgtaATGAAGAGGATATTGGGAGCTCTGCT
TRAV23	Forward	CGGTTCAGCAGGAATGCCtacgtaATGGACAAGATCTTAGGAGCATCATTTTTAG
TRAV24	Forward	CGGTTCAGCAGGAATGCCtacgtaATGGAGAAGAATCCTTTGGCAGCC
TRAV25	Forward	CGGTTCAGCAGGAATGCCtacgtaATGCTACTCATCACATCAATGTTGGTCTTAT
TRAV26-1	Forward	CGGTTCAGCAGGAATGCCtacgtaATGAGGCTGGTGGCAAGAGTAACTG
TRAV26-2	Forward	CGGTTCAGCAGGAATGCCtacgtaATGAAGTTGGTGACAAGCATTACTGTACTCC
TRAV27	Forward	CGGTTCAGCAGGAATGCCtacgtaATGGTCCTGAAATTCTCCGTGTCC
TRAV29	Forward	CGGTTCAGCAGGAATGCCtacgtaATGGCCATGCTCCTGGGGG
TRAV30	Forward	CGGTTCAGCAGGAATGCCtacgtaATGGAGACTCTCCTGAAAGTGCTTTCAG
TRAV34	Forward	CGGTTCAGCAGGAATGCCtacgtaATGGAGACTGTTCTGCAAGTACTCCTAGG
TRAV35	Forward	CGGTTCAGCAGGAATGCCtacgtaATGCTCCTTGAACATTTATTAATAATCTTGTGG
TRAV36	Forward	CGGTTCAGCAGGAATGCCtacgtaATGATGAAGTGTCCACAGGCTTTACTAGC

TRAV38-1	Forward	CGGTTCAGCAGGAATGCCtacgtaATGACACGAGTTAGCTTGCTGTGGG
TRAV38-2	Forward	CGGTTCAGCAGGAATGCCtacgtaATGGCATGCCCTGGCTTCCT
TRAV39	Forward	CGGTTCAGCAGGAATGCCtacgtaATGAAGAAGCTACTAGCAATGATTCTGTGG
TRAV40	Forward	CGGTTCAGCAGGAATGCCtacgtaATGAACTCCTCTCTGGACTTTCTAATTCTGA
TRAV41	Forward	CGGTTCAGCAGGAATGCCtacgtaATGGTGAAGATCCGGCAATTTTTG
TRAC External	Reverse	CAGACAGACTTGTCACTGGATTTAGAGTCTC
TCRbeta PCR reaction 1		
TRBV2	Forward	CAGAAGACGGCATACGAGATcaattgATGGATACCTGGCTCGTATGCTGG
TRBV3-1	Forward	CAGAAGACGGCATACGAGATcaattgATGGGCTGCAGGCTCCTCTG
TRBV4-1	Forward	CAGAAGACGGCATACGAGATcaattgATGGGCTGCAGGCTGCTCTG
TRBV5-1	Forward	CAGAAGACGGCATACGAGATcaattgATGGGCTCCAGGCTGCTCTGTT
TRBV5-3	Forward	CAGAAGACGGCATACGAGATcaattgATGGGCCCCGGGCTCC
TRBV5-4	Forward	CAGAAGACGGCATACGAGATcaattgATGGGCCCTGGGCTCCTCT
TRBV5-8	Forward	CAGAAGACGGCATACGAGATcaattgATGGGACCCAGGCTCCTCTTCT
TRBV6-1	Forward	CAGAAGACGGCATACGAGATcaattgATGAGCATCGGGCTCCTGTGC
TRBV6-2	Forward	CAGAAGACGGCATACGAGATcaattgATGAGCCTCGGGCTCCTGTG
TRBV6-4	Forward	CAGAAGACGGCATACGAGATcaattgATGAGAATCAGGCTCCTGTGCTGTG
TRBV6-6	Forward	CAGAAGACGGCATACGAGATcaattgATGAGCATCAGCCTCCTGTGCTG
TRBV7-1	Forward	CAGAAGACGGCATACGAGATcaattgATGGGCACAAGGCTCCTCTGC
TRBV7-2	Forward	CAGAAGACGGCATACGAGATcaattgATGGGCACCAGGCTCCTCTTCT
TRBV7-3	Forward	CAGAAGACGGCATACGAGATcaattgATGGGCACCAGGCTCCTCTG
TRBV7-6	Forward	CAGAAGACGGCATACGAGATcaattgATGGGCACCAGTCTCCTATGCTG
TRBV7-7	Forward	CAGAAGACGGCATACGAGATcaattgATGGGTACCAGTCTCCTATGCTGGG
TRBV7-9	Forward	CAGAAGACGGCATACGAGATcaattgATGGGCACCAGCCTCCTCTG
TRBV9	Forward	CAGAAGACGGCATACGAGATcaattgATGGGCTTCAGGCTCCTCTGCT
TRBV10-1	Forward	CAGAAGACGGCATACGAGATcaattgATGGGCACGAGGCTCTTCTTCTATG
TRBV10-2	Forward	CAGAAGACGGCATACGAGATcaattgATGGGCACCAGGCTCTTCTTCTATG
TRBV10-3	Forward	CAGAAGACGGCATACGAGATcaattgATGGGCACAAGGTTGTTCTTCTATGTG
TRBV11-1	Forward	CAGAAGACGGCATACGAGATcaattgATGAGCACCAGGCTTCTCTGCTG
TRBV11-3	Forward	CAGAAGACGGCATACGAGATcaattgATGGGTACCAGGCTCCTCTGCTG
TRBV12-3	Forward	CAGAAGACGGCATACGAGATcaattgATGGACTCCTGGACCTTCTGCTGT
TRBV12-4	Forward	CAGAAGACGGCATACGAGATcaattgATGGACTCCTGGACCCTCTGCTG
TRBV12-5	Forward	CAGAAGACGGCATACGAGATcaattgATGGCCACCAGGCTCCTCTG
TRBV13	Forward	CAGAAGACGGCATACGAGATcaattgATGCTTAGTCCTGACCTGCCTGACTC
TRBV14	Forward	CAGAAGACGGCATACGAGATcaattgATGGTTTCCAGGCTTCTCAGTTTAGTGT
TRBV15	Forward	CAGAAGACGGCATACGAGATcaattgATGGGTCCTGGGCTTCTCCACT
TRBV16	Forward	CAGAAGACGGCATACGAGATcaattgATGAGCCCAATATTCACCTGCATCA
TRBV17	Forward	CAGAAGACGGCATACGAGATcaattgATGGATATCTGGCTCCTCTGCTGG
TRBV18	Forward	CAGAAGACGGCATACGAGATcaattgATGGACACCAGAGTACTCTGCTGTGC
TRBV19	Forward	CAGAAGACGGCATACGAGATcaattgATGAGCAACCAGGTGCTCTGCTG
TRBV20	Forward	CAGAAGACGGCATACGAGATcaattgATGCTGCTGCTTCTGCTGCTTCT
TRBV24-1	Forward	CAGAAGACGGCATACGAGATcaattgATGGCCTCCCTGCTCTTCTTCTG
TRBV25-1	Forward	CAGAAGACGGCATACGAGATcaattgATGACTATCAGGCTCCTCTGCTACATGG
TRBV27	Forward	CAGAAGACGGCATACGAGATcaattgATGGGCCCCCAGCTCCTTG
TRBV28	Forward	CAGAAGACGGCATACGAGATcaattgATGGGAATCAGGCTCCTCTGTCG
TRBV29-1	Forward	CAGAAGACGGCATACGAGATcaattgATGCTGAGTCTTCTGCTCCTTCTCCT

TRBV30	Forward	CAGAAGACGGCATACGAGATcaattgATGCTCTGCTCTCTCCTTGCCCT
TRBC External	Reverse	GTGGCCAGGCACACCAGTGTG
TCR alpha PCR reaction 2		
TRAC Internal	Reverse	CAGCTGGTACAccgcggGGTCAGGGTTCTG
TRAV Adaptor	Forward	CGGTTCAGCAGGAATGCCtacgtaATG
TCR beta PCR reaction 2		
TRBC Internal	Reverse	CTCTGCTTCTGATGGttcgaaCACAGCGACCTCGG
TRBV Adaptor	Forward	CAGAAGACGGCATACGAGATcaattgATG
Sequencing primers		
pMSCV-II	Forward	CCTCCTCTTCCTCCATCCGCC
TCRbeta	Reverse	GCCAAGCACACGAGGGTAGCC
IRES	Reverse	AACGCACACCGGCCTTATTCC
*Lower case letters within the primer sequences indicate the incorporated restriction enzyme cut sites		

Table 1: Reverse transcription, PCR, and sequencing primers.

Step 2.2	**amounts per well**		
RT-PCR reaction (6µL)	**(µL)**		
10x buffer	0.6		
2x dNTP	0.24		
10% Triton X	0.06		
3 TCR-specific primers (10mM ea.)	0.23 ea. (x 3 = 0.69)		
Enzyme	0.18		
Rnase inhibitor	0.28		
NF(nuclease-free)-H2O	3.95		
Step 3.1			
a PCR reaction 1 (25µL)	**(µL)**	**b PCR reaction 1 (25µL)**	**(µL)**
5x buffer	5	5x buffer	5
dNTP (10mM)	1	dNTP (10mM)	1
DMSO	0.75	DMSO (3%)	0.75
a pool (F primer) (2.3µM)	0.3	b pool (F primer) (2.3µM)	0.5
a external R primer (10uM)	0.6	b external R primer (10µM)	1
DNA polymerase	0.2	Go Taq polymerase	0.2
RT-PCR cDNA	2.5	RT-PCR cDNA	2.5
NF-H_2O	14.65	NF-H_2O	14.05
Step 3.8			
PCR reaction 2 - same for a and b (25µL)	**(µL)**		
5x buffer	5		
dNTP (10mM)	1		
DMSO	0.75		
Adaptor F primer (10µM)	1		
Internal R primer (10µM)	1		
DNA polymerase	0.2		
PCR rxn 1 product	2.5		
NF-H_2O	13.55		

Table 2. Reverse transcription and PCR reactions.

Steps 4.2 and 4.3			
TRBV digest reaction (50µL)	**(µL)**	**mTCR-pMIA vector digest (500µL)**	**(µL)**
DNA (~20µg)		DNA (~20µg)	
MfeI	2	MfeI	20
BstbI	2	BstbI	20
10x Buffer	5	10x Buffer	50
H_2O		H_2O	
Step 4.4			
CIP reaction (100µL)	**(µL)**	DNA(~1.5mg)	
Digested & purified vector DNA	84		
10x Buffer	10		
CIP enzyme	6		
Step 4.5			
Ligation reaction (20µL)	**(µL)**		
Digested/CIPed vector DNA	(total insert and vector amount is ~150ng)		
Insert DNA	(6 molar access to vector)		
2x Ligase buffer	10		
Ligase enzyme	1		
H_2O			
Step 4.9			
Test Digest reaction (12µL)	**(µL)**		
DNA (100-300ng)	1		
MfeI	0.25		
BstbI	0.25		
10x Buffer	1.2		
H_2O	9.3		
Step 4.11			
TRAV digest reaction (60µL)	**(µL)**	**Beta containing - mTCR-pMIA vector digest (120µL)**	
DNA(~1.5µg)		DNA (~3µg)	
SnaBI	3	MfeI	6
SacII	2	BstbI	4
10x Buffer	6	10x Buffer	10
H_2O		H_2O	

Table 3. Vector cloning reactions.

Discussion

In the current protocol, we describe an efficient method for single cell TCR amplification and subsequent sub-cloning of paired TCR alpha and beta chains into a template retroviral expression vector. Although, several single cell PCR protocols have been developed, none so far have been compatible with immediate sub-cloning into an expression vector. In most cases, a partial sequence encompassing the highly variable CDR3 regions is amplified, with enough sequence to extrapolate the variable region. This smaller PCR product size supports higher PCR efficiency, but necessitates *de novo* TCR gene synthesis for functional studies. The current protocol maintains the efficiency of previously published CDR3-focused single cell TCR sequencing protocols, while at the same time yielding a longer amplicon containing the whole variable region suitable for cloning. Therefore, the system described has an increased flexibility in giving a quantitative sequence, as well as functional output.

Depending on the scientific question addressed, the source and specificity of T cells analyzed can vary. However, if the end goal of the project is the analysis of the *in vivo* TCR function, it is necessary to know the HLA restriction of isolated T cells. The current protocol has been specifically designed to be compatible with *in vivo* TCR expression in HLA-humanized mice. Many of these mice have been generated and are now commercially available, including HLA-A2.1, HLA-DQ8 (HLA-DQA1*301,HLA-DQB1*302), HLA-DQ6 (DQA1*0102,HLADQB1*0602), HLA-DR4

(DRB1*0401), HLA-DR1 (DRA*0101, DRB1*0101), HLA-A11 (A*11:01/K), HLA-B2705, HLA-A24, and HLA-B*0702 transgenic strains. This protocol can be successfully combined with the previously published protocol for retroviral mediated bone marrow stem cell expression of human TCRs[4,15,16]. We expect that the humanized TCR retrogenic system will be applicable and highly useful for functional analysis of human TCRs in various autoimmune, cancer, and infectious models.

The current single cell PCR protocol describes the critical steps and gives suggestions necessary for successful amplification of paired TCR-alpha and TCR-beta sequences. The first critical step is a timely performance of cDNA transcription, and subsequent first round of multiplex PCR. All the steps involved should be performed in a timely manner, and all the reagents and cells kept on ice to prevent RNA and cDNA degradation. Secondly, because the protocol is designed to be highly sensitive to low levels of the template, it is highly susceptible to contamination. Therefore, it is imperative to work in a template-free area, away from amplified PCR products or TCR vector cloning. A separate room or hood should be used to set up cDNA and PCR reactions. Template from the first PCR reaction should be added in a different area from where the PCR is set up. It is advisable that a separate set of aliquoted reagents be used for the single cell PCR experiments.

This protocol has been specifically optimized using the reagents listed in **Table of Materials**, and any substitutions in reagents will require additional optimization. The initial identification of antigen-specific cells can be modified to use peptide-MHC tetramers. Tetramer staining allows for simultaneous assessment of antigen specificity, as well as HLA-restriction. Alternative methods for the detection of antigen reactivity, such as upregulation of early activation markers or secretion of inflammatory cytokines can be considered in the absence of tetramer reagents. For example, dual specificity fusion antibodies specific for a T cell antigen and IFNγ can be combined with magnetic bead isolation to enrich for antigen reactive IFNγ producing cells.

While the chimeric human/mouse construct described in our protocol ensures compatibility with mouse cells, the level of its compatibility with human CD3 complex is unknown. Although not formally tested by us, others have shown that chimeric TCR constructs with murine constant regions can be expressed on the surface of human lymphocytes[17]. This indicates that murine TCR constant regions can support interaction with the human CD3 signaling complex. However, if the goal is to express the identified TCRs in human primary cells or human cell lines, such as Jurkat cells, a more optimal approach may be to use a fully human construct. This can be accomplished by swapping the murine constant regions within the vector with human constant regions.

If an identified TCR variable region contains one of the restriction sites used for cloning, the TCR should be inserted into a shuttle vector and subsequently mutated using a site directed mutagenesis kit to induce a silent nucleotide change within the restriction cut site. An alternative approach is *de novo* synthesis of the TCR variable region of interest modified to exclude the unwanted restriction site.

The main limitation of this technique is the inability to eliminate amplification of secondary 'cytoplasmic' alpha chains. TCR-alpha genes do not go through allelic exclusion like TCR-beta genes, thus a significant proportion of T cells will express a secondary 'cytoplasmic' alpha chain[14]. The described PCR protocol only results in amplification of one TCR alpha chain, but if the amplified alpha chain is the 'cytoplasmic' alpha chain it may not pair with the amplified beta chain. Therefore, it is imperative to test TCR cell surface expression in HEK293T cells to ensure successful chain pairing.

Disclosures

The authors have nothing to disclose.

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References

1. Germain, R.N. T-cell development and the CD4-CD8 lineage decision. *Nat Rev Immunol.* **2** (5), 309-322 (2002).
2. Singer, A., Adoro, S., Park, J.H. Lineage fate and intense debate: myths, models and mechanisms of CD4- versus CD8-lineage choice. *Nat Rev Immunol.* **8** (10), 788-801 (2008).
3. Tubo, N.J. *et al.* Single naive CD4+ T cells from a diverse repertoire produce different effector cell types during infection. *Cell.* **153** (4), 785-796 (2013).
4. Sprouse, M.L. *et al.* Rapid identification and expression of human TCRs in retrogenic mice. *J Immunol Methods.* (2016).
5. Lennon, G.P. *et al.* T cell islet accumulation in type 1 diabetes is a tightly regulated, cell-autonomous event. *Immunity.* **31** (4), 643-653 (2009).
6. Dash, P. *et al.* Paired analysis of TCRalpha and TCRbeta chains at the single-cell level in mice. *J Clin Invest.* **121** (1), 288-295 (2011).
7. Kobayashi, E. *et al.* A new cloning and expression system yields and validates TCRs from blood lymphocytes of patients with cancer within 10 days. *Nat Med.* **19** (11), 1542-1546 (2013).
8. Szymczak, A.L. *et al.* Correction of multi-gene deficiency in vivo using a single 'self-cleaving' 2A peptide-based retroviral vector. *Nat Biotechnol.* **22** (5), 589-594 (2004).
9. Szymczak, A.L., Vignali, D.A. Development of 2A peptide-based strategies in the design of multicistronic vectors. *Expert Opin Biol Ther.* **5** (5), 627-638 (2005).
10. Bettini, M.L., Bettini, M., Vignali, D.A. T-cell receptor retrogenic mice: a rapid, flexible alternative to T-cell receptor transgenic mice. *Immunology.* **136** (3), 265-272 (2012).

11. Holst, J., Vignali, K.M., Burton, A.R., Vignali, D.A. Rapid analysis of T-cell selection in vivo using T cell-receptor retrogenic mice. *Nat Methods.* **3** (3), 191-197 (2006).
12. Mannering, S.I. *et al.* A sensitive method for detecting proliferation of rare autoantigen-specific human T cells. *J Immunol Methods.* **283** (1-2), 173-183 (2003).
13. James, E.A., LaFond, R., Durinovic-Bello, I., Kwok, W. Visualizing antigen specific CD4+ T cells using MHC class II tetramers. *J Vis Exp.* (25) (2009).
14. Corthay, A., Nandakumar, K.S., Holmdahl, R. Evaluation of the percentage of peripheral T cells with two different T cell receptor alpha-chains and of their potential role in autoimmunity. *J Autoimmun.* **16** (4), 423-429 (2001).
15. Lee, T., Shevchenko, I., Sprouse, M.L., Bettini, M., Bettini, M.L. Retroviral Transduction of Bone Marrow Progenitor Cells to Generate T-cell Receptor Retrogenic Mice. *J Vis Exp.* (113) (2016).
16. Bettini, M.L., Bettini, M., Nakayama, M., Guy, C.S., Vignali, D.A. Generation of T cell receptor-retrogenic mice: improved retroviral-mediated stem cell gene transfer. *Nat Protoc.* **8** (10), 1837-1840 (2013).
17. Cohen, C.J., Zhao, Y., Zheng, Z., Rosenberg, S.A., Morgan, R.A. Enhanced antitumor activity of murine-human hybrid T-cell receptor (TCR) in human lymphocytes is associated with improved pairing and TCR/CD3 stability. *Cancer Res.* **66** (17), 8878-8886 (2006).

